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(57) Abstract

Novel ricin molecules having ricin-type activity and targeted toxin conjugates containing the same are disclosed. The molecules are characterized by modification within the lectin binding regions of the B chain. Methods for making these molecules and conjugates are disclosed as are the therapeutic compositions containing the same.

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# IMPROVED RICIN MOLECULES AND RICIN TOXIN CONJUGATES

## Background of the Invention

The present invention relates to novel ricin molecules having ricin-type activity and conjugates comprised of these novel ricin molecules linked to selected ligands. More particularly, it relates to the production of ricin-type substances using recombinant DNA techniques, and the therapeutic use of conjugates comprised of these novel ricin molecules linked to a selected ligand.

The plant toxin ricin is a well known molecule consisting of two polypeptide subunits, designated the A and B chains or subunits. The A chain is believed to provide the catalytic (toxic) activity, while the B chain is believed to provide both cell surface binding affinity (lectin activity) as well as translocation activity permitting the A chain of ricin to be translocated across the cell membrane into the cytosol. [See, e.g., R.J. Youle and D.M. Neville, J. Biol. Chem. 257: 1598-1601 (1982)]. The toxicity is believed to be effected by enzymatic action on 28S rRNA in the ribosomal 60S subunit of the ribosomal particle leading to inhibition of protein synthesis. [See, e.g., Y. Endo et al. J. Biol.

Chem. 262: 5908-5912, (1987)]. Ricin, like many toxins, is not cell specific because the cell binding domain recognizes galactosyl residues of glycoproteins and glycolipids present on the surface of many cell types.

Two different forms of ricin, known as ricin D and ricin E, have been characterized [T. Araki and G. Funatsu, Biochim. Biophys. Acta 911: 191-200 (1987) and B.F. Ladin et al, Plant Molecular Biology 9: 287-295 (1987)]. Both are equally toxic to animals, but only ricin D toxin binds to galactose containing supports. By comparison of their amino acid sequences with that of the closely related agglutinin, it appears that ricin E is the result of a genetic recombination between the ricin D and agglutinin genes in the region coding for the B chain subunit. In all isoforms of the protein the A and B chains are linked by a single, comparatively stable, disulfide bond. They are initially formed as a prepro-like molecule consisting of a contiguous ca. 550 amino acid protein with a putative signal peptide and short fragment linking the A and B chains. During processing of the primary gene product into the mature protein, an interdisulfide bond is formed between the two subunits, four intradisulfide bonds are probably formed in the B subunit, and through proteolytic action the signal peptide and the amino acid linker are eliminated [F.I. Lamb. et al, Eur. J. Biochem., 148: 265-270 (1985)].

A comparison of the primary protein sequence of the B chain subunit of ricin D and the x-ray crystallographic structure indicates that the subunit is a product of at least two gene duplications [E. Rutenber et al., Nature, 326: 624-626 (1987) and W. Montfort et al., J. Biol. Chem., 262: 5398-5403, (1987)]. The B chain subunit appears to consist of two domains, herein defined as Domain I (amino acid 1-135) and Domain II (amino acid 136-262). Further examination of the primary and tertiary structure suggests that each domain can be divided into four homologous sub-domains or regions. These regions are defined as 1$\lambda$ (amino acid 1-16), 1$\alpha$ (amino acid 17-59), 1$\beta$ (amino acid 60-100) and 1$\gamma$ (amino acid 101-135) in Domain I and 2$\lambda$ (amino acid 136-147), 2$\alpha$ (amino acid 148-183), 2$\beta$ (amino acid 184-226), and 2$\gamma$ (amino acid 227-262) in Domain II.

Ricin and other toxins have been employed in conjugates which consist of a toxin molecule or a part thereof linked to a ligand such as a growth factor or antibody; the latter confer cell specificity. [See, e.g. U.S. Patent 4,340,535; U.S. Patent 4,359,457; and U.S. Patent 4,664,911]. Conjugates consisting of a toxin moiety and an antibody are well known in the art as "immunotoxins" or ITs. [See, e.g. I. Pastan et al, Cell 47: 641-648 (1986)]. Such conjugated molecules can be used as targeted or cell specific therapeutics in the treatment of various forms of cancer.

The ligand moiety of the toxin conjugate binds to its receptor or antigenic determinant on the cell surface and the conjugate is internalized into the cell, for example by endocytosis. Once within the cell, the toxin moiety is presumably released into the cytosol from the endocytic vesicle or receptosome.

The non-specific cell binding properties of the B chain of ricin may be altered or removed in a variety of ways. In U.S. Patent 4,340,535 the B chain is eliminated entirely as the toxin moiety of the conjugate is comprised of only the A chain of ricin. In U.S. Patent 4,359,457 the ricin galactose site is blocked with lactose, thereby preventing non-specific binding to galostose residues present on the surface of many cell types. Alternatively, B chain has been linked to an antibody against the constant region of the antibody moiety in a ricin A chain immunotoxin, or against the same antigenic determinant on the target cell. [E.S. Vitetta, et al, J. Exp. Med., 160: 341-346 (1984); and E.S. Vitteta, et al, PNAS 80: 6332-6335, (1983)].

Many of these conjugated molecules known in the art may be faced with significant shortcomings. For example, those conjugates containing only the A chains may be more specific, but less active than the intact toxin molecule, presumably as a result of loss or diminution of the translocation function associated with the B chain. [See, e.g. Weil-Hillman et al, Cancer Res. 45: 1328-1336 (1985)]. Another difficulty may

arise with conjugates wherein conjugation between the toxin and ligand is effected by disulfide bond formation. Disulfide bonds of this type are generally not stable in blood and other tissue fluids and therefore may be disrupted before reaching the intended target. [N.L. Letvin et al, J. Clin. Invest. 77: 977-984 (1986)]. Furthermore, freed targeting agent may then compete with intact conjugates for the cell surface marker.

## Summary of the Invention

Novel ricin molecules have now been discovered. The new molecules have an amino acid sequence substantially similar to the amino acid sequence of native D ricin molecules wherein the lectin-binding domains of the B chain are modified or "engineered" to alter or remove the non-specific cell binding function. It is contemplated however that the modifications do not alter the B chain translocation activity and therefore, novel toxin conjugates of the invention containing a modified B chain are both active and specific. In each of the embodiments described herein the amino acid sequence is the same or substantially the same as that of naturally occurring ricin D modified by the specific changes recited therein. The specific modifications are set forth

below in the Description.

The ricin molecules of the invention include analogs of ricin characterized by the various modifications or combinations of modifications as disclosed herein, which may also contain other variations, e.g. allelic variations, or additional deletion(s), substitution(s) or insertion(s) of amino acids which still retain ricin-type activity, so long as the DNA encoding these proteins (prior to the modification of the invention) is still capable of hybridizing to a DNA sequence encoding ricin under stringent conditions. [See, T. Maniatis et al., Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory (1982), pp. 387-389].

Native ricin B chain is contains two N-linked glycosylation sites. It is further contemplated that by choice of expression systems or site-specific mutagenesis techniques, the carbohydrate composition at these sites can be eliminated or modified to favorably affect the in vivo clearance of this molecule or its derivatives.

The invention further features that modified ricin B chains produced in a more homogeneous form than modified B chains not produced in cells transfected with the ricin B chain gene.

Another aspect of the invention includes DNA sequences encoding the amino acid sequences of the ricin molecules of the invention and vectors containing these sequences in operative association with an expression control sequence.

The ricin molecules of the invention are produced by the expression of the DNA molecules encoding the amino acid sequences in host cells transformed with said DNA. These host cells include mammalian, yeast, insect, fungal or bacterial cells.

The invention further features targeted toxin conjugates comprising a modified ricin B chain of the invention linked to ricin A chain and a selected ligand. Preferred ligands include, but are not limited to, antibodies, growth factors, hormones and other cell surface binding agents. The conjugate is capable of crossing the membrane of the cell bearing the receptor, antigen or oncogene for the selected ligand and acting within the cell to destroy the cell. The conjugates containing modified ricin B chains of the invention may be prepared by gene fusions or as described below.

Another aspect of the invention provides pharmaceutical compositions comprising an effective amount of such ricin conjugates. These pharmaceutical compositions may be employed in the treatment of any number of medical conditions including cancer according to the selected ligand. The selected ligand directs the composition to its target and the composition acts by attaching to the receptor, antigen or oncogene or other recognition site for the selected ligand and delivers the ricin through the cell membrane, where the toxin destroys the cell. It is contemplated that the toxin

conjugates are stable in serum and other tissue fluids and not until the conjugate enters the cytosol is the toxic moiety released.

A further aspect of the invention, therefore, is a method for treating cancer and any number of medical conditions against which the ligand is directed, by administering to a patient a therapeutically effective amount of the conjugate in a suitable pharmaceutical carrier.

The vectors and transformed cells of the invention are employed in a novel process for producing the recombinant ricin molecules of the invention. This process of production includes culturing selected cells capable of producing the ricin molecules to obtain conditioned medium and purifying the molecules therefrom.

Another aspect of the present invention provides a novel process for the production of the targeted ricin conjugates. It is contemplated that this method will provide conjugates that are significantly more stable in vivo and as active as those prepared by standard methodologies. [See e.g. A.J. Cumber et al, Methods in Enzymol. 112: 207-225 (1985)]. The process consists of attaching a peptide crosslinker to the modified ricin B chain molecule of the invention and reforming the holotoxin by disulfide bond formation with ricin A chain. The B chain of the modified holotoxin is then covalently linked as described below to the selected ligand to produce the toxin conjugate. The B chain may be

covalently linked to the ligand by a number of linkage chemistries. In a preferred embodiment, the B chain of the modified holotoxin is treated with N-succinimidyl-S-acetylthiopropionate, and reassociated with the A chain to give a holotoxin molecule. The holotoxin is treated with hydroxylamine to expose a free sulfhydryl which reacts with a maleimide-containing protein ligand. [See, I.M. Klotz and R.E. Heiney, Arch. Biochem. Biophys. 96: 606-612 (1962)]. In another embodiment, the protein ligand may contain an integral sulfhydryl group, e.g. a free cysteine or an added sulfhydryl attached to the peptide or carbohydrate portion. In another embodiment the reassociated holotoxin containing an engineered B chain is treated with N-succinimidyl 3-(2-pyridyldithio) proprionate and then crosslinked to the protein ligand by standard methods. Other crosslinkers may also be employed to covalently link the modified B chain to the selected ligand.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description of the invention, including examples of the practice thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the DNA and amino acid sequence for the ricin D gene found on EcoR1-Hind III fragment of the genomic DNA extracted from caster beans (Ricinus communis, Zanibariensis variety).

Fig. 2 illustrates the amino acid sequence for the B chain ricin D polypeptide.

Fig. 3 illustrates a restriction map of the 4.2 Kb EcoRI - HindIII fragment containing the ricin gene.

Description of the Invention

I. Modified Ricin Molecules

The invention features modified ricin molecules which lack or have a diminished cell binding affinity. These molecules include novel analogs, derivatives, and mutants of naturally occurring ricin D wherein the B chains differ in structure from the natural molecule in that they contain modifications in the area of the protein structure responsible for the cell binding function. The invention further features ricin D, B chain molecules. The modifications are contemplated to alter or eliminate the cell binding function while retaining the translocation function. In each of the embodiments the amino acid sequence is characterized by the same or substantially the same amino acid sequence as the naturally occurring ricin D molecule modified by the specific changes recited therein.

"Characterized by the same or substantially the same as the amino acid sequence of the naturally occurring molecule" as the phrase is used herein, means the amino acid sequence encoded by a DNA sequence capable of hybridizing to the DNA sequence of naturally occurring ricin under stringent hybridization conditions. Thus the ricin molecules of the invention include analogs of ricin characterized by the various modifications or combinations of modifications as

disclosed herein, which may also contain other variations, e.g. allelic variations, or additional deletion(s), substitution(s) or insertion(s) of amino acids which still retain ricin-type activity, so long as the DNA encoding these proteins (prior to the modification of the invention) is still capable of hybridizing to a DNA sequence encoding ricin under stringent conditions. [See, T. Maniatis et al., Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory (1982), pp. 387-389].

Referring to Figure 2 wherein the amino acid sequence of the B chain is presented in standard three-letter code form and numbered 1-262, the sub-domains are 1$\lambda$ (amino acid 1-16), 1$\alpha$ (amino acid 17-59), 1$\beta$ (amino acid 60-100) and 1$\gamma$ (amino acid 101-135) in Domain I; and 2$\lambda$ (amino acid 136-147), 2$\alpha$ (amino acid 148-183), 2$\beta$ (amino acid 184-226), and 2$\gamma$ (amino acid 227-262) in Domain II.

In one embodiment the protein sequence of ricin B is characterized by changes in the amino acids of sub-domain 1$\alpha$. A preferred embodiment is characterized by deletion of amino acid 40 or its replacement with a naturally occuring amino acid other than lysine and preferrably arginine, leucine or another non-charged amino acid. In further embodiments amino acid 20 and/or 39 is/are deleted or replaced preferrably with serine. In addition, this mutant may be further modified such that two cysteines are inserted within 1-10 amino acids to either side of positions 20 and 39. A further

modification, includes replacement of a naturally occurring amino acid within the first ten amino acids to either side of positions 20 and 39 with cysteine.

In another embodiment the protein sequence of ricin B is characterized by changes to amino acids in sub-domain 2$\gamma$ . In a preferred at least one of the amino acids in positions 229, 237, 247, 248, 250, 253, and 254 are changed to Asn, Arg, Val, His, Phe or Val, Asn, and Leu, respectively, to match the sequence found in ricin E. In another embodiment the last 8 to 34 amino acids of the carboxyl terminus are deleted.

In a further embodiment the protein sequence of ricin B is characterized by changes in sub-domain 1$\alpha$ and/or sub-domain 2$\gamma$ . In one embodiment amino acid 46 and/or amino acid 255 is/are deleted or replaced with a naturally occuring amino acid which is other than asparagine. In a preferred embodiment amino acid 46 and/or 255 is/are replaced with glutamine, leucine, aspartic acid, serine or lysine.

In another preferred embodiment the modified ricin is characterized by changes in which amino acid 37 and/or 248 is/are deleted or replaced with phenylalanine and/or alanine, histidine, or other non-aromatic amino acids.

In another embodiment the ricin molecule of the invention amino acid 22 and/or 234 is/are deleted or replaced with a naturally occuring amino acid other than asparatic acid. In a preferred embodiment amino acid 22 and/or 234

is/are replaced with asparagine, glutamic acid, alanine another non-charged amino acid.

In another embodiment at least one amino acid of the tripeptide sequence aspartic acid, valine and arginine at positions 22-24 and 234-236 is deleted or replaced in further embodiments. Another preferred embodiment includes an amino acid sequence wherein at least one of the prolines of positions 38 and 249 are deleted or replaced with a different naturally occurring amino acid.

Another embodiment features the insertion of 1-5 additional prolines within the region extending from amino acid 20 to 49 and from amino acid 234 to 258, or replacement with proline of one to five amino acids in the region extending from amino acid 20 to 49 and 234 to 258, or the substitution of one to ten amino acids in the region extending from amino acid 20 to 49 and amino acid 234 to 258 with a branched chain amino acid, such as leucine and isoleucine.

Another embodiment includes an amino acid sequence in which at least one amino acid from the tripeptide sequence glutamine, leucine or isoleucine, and tryptophan of the positions 35 to 37, 47 to 49, and 256 to 258 is deleted or replaced.

Further embodiments feature swapping of amino acids from homologous sub-domains. For example, amino acids 17-59 may be replaced with amino acids 148-183. In another embodiment

amino acids 227-262 are replaced with amino acids 101-135.

In further embodiments at least one of the two consensus N-linked glycosylation sites of the B chain of the ricin molecule is modified to other than a consensus N-linked glycosylation site.

In other embodiments the amino acids from the region extending from and including amino acid 13 to 143 or the amino acids from the region extending from and including amino acid 134 to 260 are deleted.

In another embodiment at least one and no more than ten of the following amino acid changes are made: amino acids changes Leu 150 to Met, Gln 158 to Lys, Ile161 to Leu, Ser165 to Thr, Ser193 to Thr, Ser195 to Ala, Arg198 to Lys, Glu199 ti Gly, Ala210 to Val, Ser229 to Asn, and Ala237 to Arg.

In further embodiments more than one of the above described embodiments are incorporated into the modified ricin B chain molecule of the invention. The above recited mutations may be used in combination with one or more additional mutations.

II. Preparation of Modified Ricin Molecules

The DNA sequences for the ricin gene have been cloned and characterized either from poly-A selected mRNA, [See, e.g., F.I. Lamb et al, Eur. J. Biochem. 148: 265-270 (1985) and M.-S. Chang et al., PNAS 84:5640-5644 (1987)] or from genomic DNA, [See, e.g. K.C. Helling Nucleic Acids Res.

13:8091-8033 (1985)], and as described in Example I below.

The present invention provides DNA sequences encoding individual variants of this invention may be produced by conventional site-directed mutagenesis of a DNA sequence encoding ricin B chain as shown in Fig. 1 or analogs or variants thereof including, but not limited to, allelic variants, analogues, derivatives and DNA sequences capable of hybridizing thereto under stringent hybridization conditions. [Maniatis, supra]. An example of one such stringent hybridization condition is hybridization at 4xSSC at 65 degrees C, followed by washing in 0.1 x SSC at 65 degrees C for one hour.

The DNA sequence encoding proricin may also be employed [F.I. Lamb, et al supra]. Such methods of mutagenesis include the M13 system of Zoller and Smith, Nucleic Acids Res. 10:6487-6500 (1982); Methods Enzymol. 100:468-500 (1983); and DNA 3:479-488 (1984); the phenotypic selection method of T.A. Kunkel PNAS 82: 488-492 (1985); heteroduplexed DNA of B.A. Oostra et al, Nature 304: 456-459 (1983), or "cassette mutagenesis" according to S.D. Porter and M. Smith, Nature 320: 766-768, (1986) and M.D. Matteucci and H.L. Heyneker, Nucleic Acid Res. 11: 3113-3121 (1983), using existing restriction sites or restriction sites introduced by mutagenesis. It is assumed that the oligonucleotide(s) used to direct mutagenesis in the above methods can be of degenerate as well as defined DNA sequence,

to yield one or many defined mutant ricin B chains. It should be understood, of course, that DNA encoding each of the ricin molecules of this invention may be analogously produced by one skilled in the art through site-directed mutagenesis using (an) appropriately chosen oligonucleotide(s).

Modification of one or both of the glycosylation sites is carried out by amino acid substitution or deletion at the asparagine-linked glycosylation recognition site present in the sequences. The asparagine-linked glycosylation recognition sites comprise tripeptide sequences which are specifically recognized by appropriate cellular glycosylation enzymes. These tripeptide sequences are either asparagine-X-threonine or asparagine-X-serine, where X is usually any amino acid. A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation recognition site (and/or amino acid deletion at the second position) results in non-glycosylation at the modified tripeptide sequence.

The invention also provides vectors for use in the method of expression of the novel ricin molecules. In order to express the modified ricin B chain of the invention, the DNA encoding the modified molecule is transferred into an appropriate expression vector and introduced into selected host cells by conventional genetic engineering techniques.

Preferably the vectors contain the full novel DNA

sequences described above which code for the novel ricin molecules of the invention. Further components of the vectors, e.g. replicons, selection genes, enhancers, promoters, and the like may be obtained from natural sources or synthesized by procedures within the knowledge of those skilled in the art. Additionally, the vectors also contain appropriate expression control sequences permitting expression of the ricin B chain polypeptide sequences. The vectors may also contain DNA sequences encoding polypeptide pre- or prepro- sequences, to allow secretion of the novel ricin molecules, from suitable host cells into the medium. [See, e.g., M.S. Chang et al, supra]. The vectors may contain selected regulatory sequences in operative association with the DNA coding sequences of the invention which are capable of directing the replication and expression thereof in selected host cells. Useful regulatory sequences for such vectors are known to one of skill in the art and are generally selected based on the type of host cells. Such selection is routine and does not form part of the present invention. A useful vector for the expression of B chain ricin is described by M.S. Chang et al, supra.

One skilled in the art can construct mammalian expression vectors for use in the invention by employing the DNA sequences of the invention and known vectors, such as pCD [Okayama et al., Mol. Cell Biol., 2:161-170 (1982)] and pJL3, pJL4 [Gough et al., EMBO J., 4:645-653 (1985)]. The

transformation of these vectors into appropriate host cells, for example the monkey COS-1 cell line, can result in expression of the ricin molecules of the invention.

One skilled in the art could manipulate the DNA sequence eliminating or replacing the mammalian regulatory sequences flanking the coding sequence with bacterial sequences to create bacterial vectors for intracellular or extracellular expression by bacterial cells. For example, the coding sequences could be further manipulated for bacterial expression as is known in the art. Preferably the sequence is operably linked in-frame to a nucleotide sequence encoding a secretory leader polypeptide permitting bacterial expression, secretion and processing of the mature variant protein as is known in the art. The compounds expressed in bacterial host cells may then be recovered, purified, and/or characterized with respect to physiochemical, biochemical, and/or clinical parameters, by known methods. The sequence could then be inserted into a known bacterial vector using procedures such as described in T. Taniguchi et al., PNAS 77:5230-5233 (1980). This exemplary bacterial vector could then be transformed into bacterial host cells and ricin B chain expressed thereby.

Similar manipulations can be performed for the construction of an insect vector [see, e.g. procedures described in published European Patent Application 155,476] for expression in insect cells.

Yeast vectors can also be constructed employing yeast regulatory sequences for intracellular or extrac llular expression of the molecules of the present invention by yeast cells. [See, e.g., procedures described in published PCT application WO86/00639 and European Patent Application EPA 123,289].

A method for producing high levels of the molecules of the invention from mammalian cells involves the construction of cells containing multiple copies of the gene. The heterologous gene can be linked to an amplifiable marker, e.g. the dihydrofolate reductase (DHFR) gene for which cells containing increased gene copies can be selected for propagation in increasing concentrations of methotrexate (MTX) according to the procedures of Kaufman and Sharp, J. Mol. Biol., 159:601-629 (1982). This approach can b employed with a number of different cell types. For example, a plasmid containing a DNA sequence for a ricin molecule of the invention in operative association with other plasmid sequences enabling expression thereof and the DHFR expression plasmid pAdA26SV(A)3 [Kaufman and Sharp, Mol. Cell. Biol., 2:1304 (1982)] and derivatives thereof can be co-introduced into DHFR-deficient CHO cells, DUKX-BII, by calcium phosphate are selected for growth in alpha media with dialyzed fetal calf serum, and subsequently selected for amplification by growth in increasing concentrations of MTX (sequencial steps in 0.02, 02, 1.0 and 5uM MTX) as described

in Kaufman et al., Mol Cell Biol., 5:1750 (1983). Transformants are cloned, and biologically active ricin B chain expression is monitored by similar assay systems described in Chang et al., supra. It is contemplated that ricin expression increases with increasing levels of MTX resistance.

The present invention also provides a method for producing the ricin molecules. The method involves culturing a suitable cell or cell line, which has been transformed with a DNA sequence coding for a ricin molecule of the invention under the control of known regulatory sequences. Suitable cells or cell lines for expression of the novel molecules may be mammalian cells, such as Chinese hamster ovary cells (CHO), monkey COS-1 cells or CV-1 cells. The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. [See, e.g., Gething and Sambrook, Nature, 293:620-625 (1981), or alternatively, Kaufman et al, Mol. Cell. Biol., 5(7):1750-1759 (1985) or Howley et al, U.S. Patent 4,419,446]. Other mammalian host cells include particularly primate cell lines and rodent cell lines, including transformed cell lines. Normal diploid cells, cell strains derived from in vitro culture of primary tissue, as well as primary explants, are also suitable. Other suitable mammalian cell lines include but are not limited to, HeLa, mouse L-929 cells, 3T3 lines derived from

Swiss, Balb-c or NIH mice, BHK or HaK hamster cell lines.

Bacterial cells are suitable hosts. For example, the various strains of E. coli (e.g., HB101, MC1061) are well-known as host cells in the field of biotechnology. Various strains of B. subtilis, Pseudomonas, other bacilli and the like may also be employed in this method.

Many strains of yeast cells known to those skilled in the art are also available as host cells for expression of the polypeptides of the present invention. Additionally, where desired, insect cells may be utilized as host cells in the method of the present invention. [See, e.g. Miller et al, Genetic Engineering, 8:277-298 (Plenum Press 1986) and references cited therein].

Stable transformants are screened for expression of the product by standard immunological or functional assays. The expressed compound is recovered, purified , and/or characterized with respect to physiochemical, biochemical and/or clinical parameters, all by known methods.

The lectin binding properties of the mutant forms of ricin B chain can be examined in several ways. For example, dimunition or elimination of the non-specific cell-binding function of the expressed mutant may be assayed by the inability of a mutant, from conditioned media or purified material, to bind to asialofetuin either free in solution or bound to a solid support, e.g. sepharose, or to acid-treated sepharose [M. S. Chang et al, PNAS 84: 5640-5644, (1987) and

T. Mise et al, Agric. Biol. Chem., 41: 2041-2046, (1977)].

Further, potentiation of toxicity by the mutant forms of ricin B chain on ricin A chain containing toxin conjugates can be examined in two formats. Firstly, by addition of excess mutant B chain to the target cells in the presence of the A chain conjugate, [R. J. Youle et al, J. Biol. Chem., 257: 1598-1601, (1982) and D. P. McIntosh et al, Fed. Eur. Biochem. Soc., 164: 17-20, (1982)]. Alternatively, the mutant B chain can be linked to an antibody against the ligand of the ricin A chain toxin conjugate, or to the same ligand as in the A chain conjugate, [R. J. Fulton et al, J. Immunol., 136: 3103-3109, (1986)]. Equally, the modified B chain can be linked directly to ricin A chain and the selected ligand and tested against the target cell.

III. Preparation of Toxin Conjugates

Having expressed and isolated the ricin B chain of the invention, the toxin conjugate is then prepared. A selected peptide crosslinker is attached to the isolated ricin B chain. One preferred cross-linker is S-acetyl protected thiopropionic acid. Ricin A chain is then added to the ricin B chain to reform the holotoxin by disulfide bond formation. Though not limited thereby, the A chain can be a recombinant A chain, natural A, a natural mutant, chemically deglycosylated ricin A or other variant A chain. [See, e.g. U.S. 4,689,401]. The modified holotoxin is then covalently

linked to a selected ligand.

The ligand to which the holotoxin is linked is not limited by the invention. The ligand is selected according to the target to which the conjugate is to be directed. The ligand may consist of growth factors such as Il-1$\alpha$,$\beta$, Il-2, Il-3, Il-4, Il-5, Il-6, M-CSF, G-CSF, GM-CSF, FGF, TGF$\alpha$, $\beta$ and TNF. The ligand may also be an antibody including monoclonal antibodies directed to a variety of epitopes on a target site, including those associated with tumor cells, virus, fungi, or bacteria. Such antibodies include, but are not limited to, NR-CO 1-5 for colon cancer and R24 for melanoma.

In a preferred method for preparation of the toxin conjugate the B chain is treated with N-succinimidyl S-acetylthiopropionate [N. Fujii, Chem. Parm. Biol. 33:362 (1985)]. The ricin A chain is then reassociated with the functionalized B chain to form the holotoxin. The S-acetyl protecting group is then cleaved with hydroxylamine or hydrazine [Klotz and Heiney, supra]. A protein ligand containing an integral sulfhydryl group or more preferrably an added maleimide or sulfhydryl group attached to the peptide or carbohydrate portion is then coupled to the B chain of the holotoxin.

In another embodiment the B chain is treated with N-succinimidyl 3-(2-pyridyldithio) propionate followed by dithiothreitol. The A chain and ligand are then added as

described by the st ps of the procedure described above. Of course, treatment with other standard crosslinkers is within the scope of the invention.

In other embodiments, where the modifications have been made to a proricin molecule, the proricin is treated proteolytically resulting in an A chain that is releasable under reductive conditions. The ligand is then linked to the ricin molecule as described above.

A ricin toxin conjugate of the present invention has application in numerous medical conditions. Depending on the condition an appropriate ligand is selected which will direct the ricin moiety to the appropriate site. The ligand imparts specificity to the conjugate molecule. Possible applications of the toxin conjugates of the invention include treatment of cancer using conjugates employing an antibody ligand directed to the cell surface of tumors. For instance, the conjugates can be used in the treatment of leukemia, lymphoma and localized cancer such as ovarian and breast carcinoma. The conjugate is internalized into the cell where it is contemplated that the ricin moiety is released thereby destroying the cell. There are several possible applications depending on the availability of the types of specific antibodies and growth factors and other ligands which may comprise the ligand moiety of the conjugate.

Therefore, as yet another aspect of the invention therefore includes a therapeutic method and composition for treating conditions such as those described above. Such a composition comprises a therapeutically effective amount of at least one of the ricin toxin conjugates of the invention. These conjugates according to the present invention may be present in a therapeutic composition in admixture with a pharmaceutically acceptable vehicle or matrix. Further therapeutic methods and compositions of the invention comprise a therapeutic amount of a ricin conjugate of the invention with a therapeutic amount of at least one other ricin conjugate of the invention. Additionally, the ricin conjugates according to the present invention or a combination of the conjugates of the present invention may be co-administered with other agents beneficial to the treatment in question. The preparation of such physiologically acceptable protein compositions, having due regard to pH, isotonicity, stability and the like, is within the skill of the art.

The therapeutic method includes administering the conjugate to the patient in admixture with a pharmaceutically acceptable carrier. When administered, the therapeutic composition for use in this invention is, of course, in a pyrogen-free, physiologically acceptable form. Further, the composition may desirably be encapsulated or injected in a viscous form for delivery.

The dosage regime will be determined by the attending physician considering various factors which modify the action of the particular conjugate, e.g. the type of condition being treated, the patients age, sex, and diet, the severity of any infection, time of administration and other clinical factors. The addition of other factors to the final compoistion may also effect the dosage.

The following examples illustrate practice of the present invention in the production of ricin molecules and toxin conjugates containing the same.

EXAMPLE I

Production of Modified Ricin B Chain

Genomic DNA is extracted from castor bean (Ricinus communis var. Zanibariensis) seedling leaves according to E.L. Sheldon In: Maize for Biological Research ed. W.F. Sheridan University Press, Grand Forks, pp. 197-202 (1982). The presence of the 4.2kb fragment was confirmed by Southern analysis of total genomic DNA digested with HindIII and/or EcoRI using the oligonucleotide probes described below. The 4.2kb EcoRI-HindIII fragment containing the ricin gene [K.C. Helling et al supra] was obtained by the restriction fragment enrichment procedure, according to R.D. Nicholls et al, Nucleic Acid Res. 13: 7569-7578 (1985), using the restriction enzymes EcoRI and HindIII. The DNA obtained from the 4.2kb size range was cloned into pUC18 digested with the same

enzymes. After transformation of the DNA into the host JM107 mcrB⁻ (ER1451, New England BioLabs) the library was screened with oligonucleotides #1 and #2 (Table 1) derived from previously published sequences for the ricin gene [K.C. Helling et al supra and F.I. Lamb et al supra]. The oligonucleotides hybridize specifically to the sections of the ricin gene encoding A and B chains, respectively. One double positive clone from a library of ca. 30,000 transformants was obtained and was designated pRICB.

Figure 3 illustrates a restriction map of the 4.2kb EcoRI-HindIII fragment containing the ricin gene. In the following description the information in brackets corresponds to the designation in Figure 3. In particular, in Figure 3 | | represents restriction fragments described herein below; ----- represents deleted sequences; and ~~~~~ represents the pUC18 sequence. The oligonucleotides referenced in the description which follows are set forth in Table I.

Referring to Figure 3, the 'parental' plasmid, pRAB, is constructed to facilitate the cloning of the B chain gene fragment into various expression systems and for subsequent mutagenesis. This plasmid is constructed by replacing the KpnI-BamHI fragment ([K-BH] Fig. 3, fragment A) in pRICB with two oligonucleotides, #3 and #4, reforming the two restriction sites, and introducing a PstI [P] site at th first codon of the B chain section. Subsequently, in the

above plasmid (pRICB5'), the NcoI-HindIII [N-H] fragment (Fig. 3, fragment B) is replaced by two oligonucleotides, #5 and #6, reforming the above sites and

Table I

#1 GGTTTATTTTAGTTGAACTCTCAAATCATGCAGAGCTTTCTGTTAC

#2 AAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACTAATAA

#3 CAAATTCTGCAGCTGATGTTTGTATG

#4 GATCCATACAAACATCAGCTGCAGAATTTGGTAC

#5 CATGGTGACCCAAACCAAATATGGTTACCATTATTCTAGAAGACAGATTACTCTCTT GCAGCTCGAGTGTCCTGCAGA

#6 AGCTTCTGCAGGACACTCGAGCTGCAAGAGAGTAATCTGTCTTCTAGAATAATGGTA ACCATATTTGGTTTGGGTCAC

#7 ATGGATCCGAGCCCAT

#8 ACAAAGGGTCCCTGGCAAAATGAGGGCCAGAGCAAGCAGCACCCCGGCAAATCTGGC AGGAATCATGGTGGCTGCA

#9 GCCACCATGATTCCTGCCAGATTTGCCGGGGTGCTGCTTGCTCTGGCCCTCATTTTGC CAGGGACCCTTTGT

#10 GATCCGAGCCTTAAACAAATCATTGTTCACCCTTTCCATGGTAACCTAAACCAAATAT GGTTACCATTATT

#11 CTAGAATAATGGTAACCATATTTGGTTTAGGTTACCATGGAAAGGGTGAACAATGATT TGTTTAAGGCTCG

#12 CATTCTTCTGCCTCTCC Tyr248 to Leu

#13 CCGAGCCTTAAACAAATCATT^TGGTTACCATTATTCTAGA ^247-257

#14 AATTTGTATAGTGGGTTGGTGTTA^GCATCGGATCCGAGCCTTAAACAA ^234-236

introducing a XbaI site [Xb] at the termination codon (TAG) as well as PstI [P] and XhoI [X] sites, yielding pRAB.

For the purposes of mutagenesis the PstI-XbaI [P-Xb] fragment of pRAB (Fig. 3, fragment C) was cloned into the Rf form of M13mp18 also restricted with PstI and XbaI. To facilitate subsequent cloning steps, an AvaI [A] site was introduced between the PstI and the BamHI sites (Fig. 3) by site-directed mutagenesis using oligonucleotide #7; yielding mp18B.

The mammalian expression plasmid pSHvB contains the coding sequence for the pre-polypeptide of von Willebrand's Factor (vWF) [See e.g. PCT publication WO86/06096] followed by the coding sequence for ricin B chain. This plasmid was prepared by enzymatically joining the PstI-XbaI fragment from mp18B (the PstI cohesive end had been removed with the large fragment of DNA Polymerase I) to pSHIL-3-1 restricted with PstI-XbaI, in the presence of oligonucleotides #8 and 9 which encode the prepolypeptide of vWF. [pSHIL-3-1 was deposited February 24, 1987 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland USA under accession number 67326]. This deposit meets the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and Regulations thereunder. [See also, PCT publication WO/00598]

As described above, mutants are produced by conventional

site-directed mutagenesis on all or part of the DNA sequence encoding ricin B chain. For example, by the method of Zoller & Smith supra, as adapted by Eckstein using the mutagenesis system devised by Amersham International, U.K., the heteroduplex approach of Oostra et al supra, and restriction fragment replacement. All DNA manipulations discussed herein, unless specifically referenced, are in accordance with T. P. Maniatis et al., Molecular Cloning: A Laboratory Manual supra).

The large fragment from pSHvB digested with KpnI-XbaI was joined to the ca. 370bp KpnI-BamHI fragment from pRAB in the presence of oligonucleotides #10 and #11. This gave a DNA sequence encoding for a ricin B chain wherein the last 22 amino acids of the carboxyl terminus are changed to match those found in ricin E.

Using the M13 system with oligonucleotide #12 and #13 the codon for Tyr248 was changed to Leu and amino acids 247-257 were deleted, respectively. The modified DNA was restricted with AvaI-XbaI and the fragment encoding the ricin B chain was cloned into the expression plasmid pSHvB, wherein the wild type sequences for ricin B chain had been removed by digestion with AvaI-XbaI and purification of the large fragment. This basic procedure was used with the relevant oligonucleotides to obtain the mutants listed in Table 1.

Ricin B chain and the mutant forms can be expressed by

transient transfection of COS-1 monkey cells or by stable transformation of Chinese hamster ovary (CHO) cells with the plasmid pSHvB. COS monkey cells were transfected with pSHvB, containing any one of the mutant forms listed in Table 1, according to H. Luthman et al, Nucleic Acid Res. 11: 1295-1308, (1983) and L. M. Sompayrac et al. PNAS 78: 7575-7578, (1981). The expression of the mutant forms in COS-1 cells was studied by radiolabeling and immunoprecipatation according to A. J. Dorner et al, J. Cell Biol. 105: 2665-2674, (1987) using a rabbit polyclonal antibody to ricin B chain.[S. Ramakrishnan et al, Biochimica Biophys Acta 719: 341-348 (1982)]. COS-1 cells were labeled with [$^{35}$S]-methionine, 100$\mu$Ci/ml, for 15 min. at 40 hr to 70 hr post-transfection, followed by a 3 hr chase in medium containing 0.1M D-galactose. Media and cell extracts after lysis were immunoprecipatated as described. Examination of the immunoprecipatates on 12% SDS-PAGE revealed one major band at 34-36 kDa in the media and cell extract, as well as a minor band at 28-30 kDa in the latter.

EXAMPLE II

Purification of Modified Ricin B Chain

The conditioned media containing engineered ricin B is diluted with water and applied to an ion exchange membrane cartridge which has been equilibrated in 50mM Na phosphate

buffer (pH 7.5). Bound protein is washed with the same buffer containing 0.1M galactose and eluted with NaCl. The eluate is loaded onto a lentil-lectin affinity column which is washed with load buffer. Specifically-bound protein is eluted with alphamethylmannopyranoside. Higher molecular weight species are removed by means of a high resolution gel filtration column.

The foregoing descriptions detail presently preferred embodiments of the present invention. Numerous modifications and variations in practice thereof are expected to occur to those skilled in the art upon consideration of these descriptions. Those modifications and variations are believed to be encompassed within the claims appended hereto.

EXAMPLE III

Preparation of an M-CSF-Ricin Toxin Conjugate

An M-CSF- engineered ricin conjugate according to the invention is prepared as follows. For example, as prepared and isolated in Example I, one mg (30umole) of engineered ricin B chain in 100mM $NaHCO_3$/0.1M lactose (2ml) is reacted with SATP (130umole) in dimethylformamide (DMF). The reaction is allowed to proceed for 5 hr at 4°C. The derivatized B chain, in phosphate buffered NaCl is freshly

reduced and then reacted with ricin A chain (130umole) which is activated by reaction with Ellman's reagent [G. L. Ellman, Arch. Biochem. Biophys. 82: 70-77, (1959)]. The functionalized holotoxin is purified by gel filtration on Sepherogel$^{tm}$ TSK-3000 high pressure liquid chromatography column.

M-CSF (35umole), produced in mammalian cells as described in PCT publication WO87/06954, in 50 mM $NaH_2PO_4$ (pH7.0)/150mM NaCl is reacted with succinimidyl 4-(N-maleimidomethyl) cyclhexane-1-carboxylate (1750umole) for 1 hr at RT. The excess crosslinking reagent is removed by gel filtration. The SATP functionalized holotoxin in the argon-sparged phosphate/NaCl buffer is treated with an equivalent volume of 20mM hydroxylamine for 30 min at 4°C, quickly passed through a gel filtration column and then immediately reacted with the maleimide functionalized M-CSF in the same buffer. After a 16 hr reaction at 4°C, the desired M-CSF-engineered ricin conjugate is obtained and purified by gel filtration on a TSK-4000 high pressure liquid chromatography column.

The foregoing descriptions detail presently preferred embodiments of the present invention. Numerous modifications and variations in practice thereof are expected to occur to those skilled in the art upon consideration of these

descriptions. Those modifications and variations are believed to be encompassed within the claims appended hereto.

What is claimed is:

1. A ricin molecule having an amino acid sequence substantially identical to that of native ricin D wherein the lectin binding function of the B chain is removed or diminished.

2. The ricin molecule of claim 1 wherein amino acids of sub-domain 1$\alpha$ are changed or removed.

3. The ricin molecule of claim 2 characterized by the deletion of the amino acid in position 40 or replacement with a naturally occurring amino acid which is other than lysine.

4. The ricin molecule of claim 3 wherein said naturally occuring amino acid is selected from the group consisting of arginine, leucine and non-charged amino acids.

5. The ricin molecule of claim 2 wherein one or both cysteine residues in positions 20 and 39 is deleted or replaced with a naturally occurring amino acid.

6. The ricin molecule of claim 5 wherein one or both cysteine residues are replaced with serine.

7. The ricin molecule of claim 5 further characterized by

the changes selected from the group consisting of

a. insertion of two cysteines within a molecular distance of 1-10 amino acids to either side of said positions 20 and 39; and

b. replacement of a naturally occurring amino acid with cysteine within a molecular distance of 1-10 amino acids to either side of said positions 20 and 39.

8. The ricin molecule of claim 1 wherein amino acids of sub-domain $2\gamma$ are changed or removed.

9. The ricin molecule of claim 8 comprising at least one of the following modifications: amino acid 229 is replaced with asparagine; 237 is replaced with argenine; 247 is replaced with valine, 248 is replaced with histidine, 250 is replaced with phenylalanine or valine, 253 is replaced with asparagine and 254 is replaced with leucine.

10. The ricin molecule of claim 8 wherein the last 8 to 34 amino acids of the carboxyl terminus are deleted or replaced with amino acids to match the amino acids found in ricin E B-chain subunit.

11. The ricin molecule of claim 1 wherein the amino acids of sub-domain $1\delta$ and/or sub-domain $2\gamma$ are changed or removed.

12. The ricin molecule of claim 11 wherein at least one of the amino acids in positions 46 and 255 is deleted or replaced with a naturally occuring amino acid.

13. The ricin molecule of claim 12 wherein said naturally occurring amino acid is selected from the group consisting of glutamine, leucine, aspartic acid, serine and lysine.

14. The ricin molecule of claim 11 wherein at least one of the amino acids of positions 37 or 248 is deleted or replaced with an amino acid selected from the group consisting of phenylalanine, alanine, histidine and non-aromatic amino acids.

15. The ricin molecule of claim 11 characterized by the deletion of the the amino acid in position 22 and/or 234 or replacement with a naturally occurring amino acid which is other than aspartic acid.

16. The ricin molecule of claim 15 wherein said naturally occurring amino acid is selected from the group consisting of asparagine, glutamic acid, alanine and non-charged amino acids.

17. The ricin molecule of claim 11 wherein at least one amino acid acid of the tripeptide sequence aspartic acid,valine and

arginine at positions 22-24 and 234-236 are deleted or replaced.

18. The ricin molecule of claim 11 wherein the at least one of the prolines of positions 38 and 249 are deleted or replaced with a different naturally occurring amino acid.

19. The ricin molecule of claim 11 wherein one to five amino acids in the region extending from amino acid 20 to 49 and 234 to 258 are replaced with proline.

20. The ricin molecule of claim 11 characterized by the substitution of one to ten amino acids in the region extending from amino acid 20 to 49 and amino acid 234 to 258 with a branched chain amino acid.

21. The ricin molecule of claim 20 wherein said branched chain amino acid is selected from the group consisting of leucine and isoleucine.

22. The ricin molecule of claim 1 wherein at least one amino acid from the tripeptide sequence glutamine, leucine or isoleucine, and tryptophan of the positions 35 to 37, 47 to 49, and 256 to 258 are deleted or replaced.

23. The ricin molecule of claim 1 wherein amino acids from

one sub-domain are replaced with amino acids from a homologous sub-domain.

24. The ricin molecule of claim 23 wherein amino acids 17-59 are replaced with amino acids 148-183.

25. The ricin molecule of claim 23 wherein amino acids 227-262 are replaced with amino acids 101-135.

26. The ricin molecule of claim 23 wherein amino acids 227-262 are replaced with amino acids 101-135 and amino acids 17-59 are replaced with amino acids 148-183.

27. The ricin molecule of claim 1 comprising at least one and no more than ten of the following amino acids changes Leu 150 to Met, Gln 158 to Lys, Ile161 to Leu, Ser165 to Thr, Ser193 to Thr, Ser195 to Ala, Arg198 to Lys, Glu199 ti Gly, Ala210 to Val, Ser229 to Asn, and Ala237 to Arg.

28. The ricin molecule of claim 1 wherein at least one of the consensus N-linked glycosylation sites of the B chain of said ricin molecule is modified to other than a consensus N-linked glycosylation site.

29. The ricin molecule of claim 1 wherein the amino acids from the region extending from and including amino acid 13

through 143 are deleted.

30. The ricin molecule of claim 1 wherein the amino acids from the region extending from and including amino acid 134 to 260 are deleted.

31. A DNA encoding said ricin molecule of claims 1-30.

32. A ricin molecule having reduced or eliminated non-specific cell binding activity produced by the expression of a DNA molecule of claim 30 in a host cell selected from the group consisting of mammalian, yeast, insect, fungal and bacterial cells.

33. A therapeutic composition comprising an effective amount of the ricin molecule of claims 1-30 linked to a selected ligand.

34. The therapeutic composition of claim 33 wherein said ligand is a growth factor.

35. The therapeutic composition of claim 34 wherein said growth factor is selected from the group consisting of Il-1$\alpha$,$\beta$, Il-2, Il-3, Il-4, Il-5, Il-6, M-CSF, GM-CSF, G-CSF, FGF, TGF$\alpha$, $\beta$ and TNF.

36. The therapeutic composition of claim 33 wherein said ligand is an antibody or a portion thereof.

37. A method of producing a ricin conjugate comprising the steps of:

(a). attaching a peptide crosslinker to the B chain molecule of claims 1-30;

(b). attaching a ricin A chain molecule to said B chain molecule by disulfide bond formation to produce a modified ricin holotoxin; and

(c). covalently linking the B chain of said modified holotoxin to a selected protein ligand.

38. The method of claim 37 wherein said crosslinker is N-succinimidyl S-acetylthiopropionate and said B chain of said modified holotixin is linked to said ligand by the process comprising

(a). treating said holotoxin with an S-acetyl cleaving agent; and

(b). coupling said holotoxin to said ligand containing a maleimide group or other sulfhydryl reactive moiety.

Figure 1

```
        10          20          30          40          50
GTCTCAGAGG ATCTTAACAA AAGAATATAA TATATAAATA ATATATTAAA

        60          70          80          90         100
GANTGCCCTA GAAAATGCAT TTACTGTACT TAAATAACCT GTTTTGCTCT

       110         120         130         140         150
TAATATTTTA TTATTTTTAT TTCTATAATA AAAAATATTT TAAGAAATAT

       160         170         180         190         200
TTAAGTATAA AAAATAAAGT ATTTTATTGA TGTCCACTGT ACTTTTTATA

       210         220         230         240         250
TTTTATTTCT TATTTTACTT TTGTTTCCAA GGGCATCAAT ATCTTTTTTT

       260         270         280         290         300
TTTCTGTTTA ATTTTTATTA ATAAAAAAAA TAATTACAAA TATTAATTAA

       310         320         330         340         350
TCAAATACAT GGAAATTTAT TTTTATAAAA AAAAATCCTT CAAATTTTTT

       360         370         380         390         400
TAAAATGTCA TTTTGACCCT AAATTTCTTT TAATAGTTAG TGTTCTAATA

       410         420         430         440         450
AAAAAAATTT ACCCAATAAT TTTTCTAATA TTTCATTATT CTTTTATAAG

       460         470         480         490         500
ACAAACTCTT AGCCTCTAGA ATTATTTTAA GGATATATAT AATTTGTCTC

       510         520         530         540         550
TCTTTCTCTT TAACATAGCC TTAGTTTCCA ATAAATAAAT AATGAAATAT

       560         570         580         590         600
ATTTCACTCT TCATTTCTTT AAACTTCTTA CATTTTTTTT TGTAGCATTC
```

Figure 1 (Con't)

```
        610         620         630         640         650
TTTGTAAGTG GAATGACAAA ACCGTTAATG ATGTTCTTTT AAAAGTGAAA

        660         670         680         690         700
GATGTTTATA TATTGCAGTA CAGATAATGA TATATCTACT GCACTACATA

        710         720         730         740         750
AAACAATTTA AATCTCCCTG TTTATTTTAA GAAGTTATAN TTTTCTTTCT

        760         770         780         790         800
TTCTCATCCT AAGAAAGTTA AATTACTGTA ATCGACATTA TATGAATTTT

        810         820         830         840         850
AACTAATTCC GTTTCTAATT TATAATTATT TCGTTAAACC AATCAATTCC

        860         870         880         890         900
CTTTAAACAC TGCTTATGCA TATTCTGTCT CAATTTATAT TGGCATGCAT

        910         920         930         940         950
CTTCCGTATT AATTTATAAG TTCATTTTTA TTGATCAAGT ATTTGTGGTT

        960         970         980         990        1000
TTCTTTATAT AAAAAATGTA TTAGTGTTTT TCTGTATTAA TTTTATAAGT

       1010        1020        1030        1040        1050
TCATCTTTAT GAGAATGCTA ATGTATTTGG ACAGCCAATA AAATTCCAAG

      1060                                1082                1097
AATTGCTGCA ATCAAAG ATG AAA CCG GGA GGA AAT ACT ATT GTA ATA
                   MET Lys Pro Gly Gly Asn Thr Ile Val Ile

                1112                1127
TGG ATG TAT GCA GTG GCA ACA TGG CTT TGT TTT GGA TCC ACC
Trp MET Tyr Ala Val Ala Thr Trp Leu Cys Phe Gly Ser Thr

1142                1157                1172
TCA GGG TGG TCT TTC ACA TTA GAG GAT AAC AAC ATA TTC CCC
Ser Gly Trp Ser Phe Thr Leu Glu Asp Asn Asn Ile Phe Pro

   1187                1202                1217
AAA CAA TAC CCA ATT ATA AAC TTT ACC ACA GCG GGT GCC ACT
Lys Gln Tyr Pro Ile Ile Asn Phe Thr Thr Ala Gly Ala Thr
```

Figure 1 (Con't)

```
        1232                1247                1262
GTG CAA AGC TAC ACA AAC TTT ATC AGA GCT GTT CGC GGT CGT
Val Gln Ser Tyr Thr Asn Phe Ile Arg Ala Val Arg Gly Arg

            1277                1292                1307
TTA ACA ACT GGA GCT GAT GTG AGA CAT GAA ATA CCA GTG TTG
Leu Thr Thr Gly Ala Asp Val Arg His Glu Ile Pro Val Leu

                1322                1337
CCA AAC AGA GTT GGT TTG CCT ATA AAC CAA CGG TTT ATT TTA
Pro Asn Arg Val Gly Leu Pro Ile Asn Gln Arg Phe Ile Leu

1352                1367                1382
GTT GAA CTC TCA AAT CAT GCA GAG CTT TCT GTT ACA TTA GCG
Val Glu Leu Ser Asn His Ala Glu Leu Ser Val Thr Leu Ala

   1397                1412                1427
CTG GAT GTC ACC AAT GCA TAT GTG GTA GGC TAC CGT GCT GGA
Leu Asp Val Thr Asn Ala Tyr Val Val Gly Tyr Arg Ala Gly

        1442                1457                1472
AAT AGC GCA TAT TTC TTT CAT CCT GAC AAT CAG GAA GAT GCA
Asn Ser Ala Tyr Phe Phe His Pro Asp Asn Gln Glu Asp Ala

            1487                1502                1517
GAA GCA ATC ACT CAT CTT TTC ACT GAT GTT CAA AAT CGA TAT
Glu Ala Ile Thr His Leu Phe Thr Asp Val Gln Asn Arg Tyr

                1532                1547
ACA TTC GCC TTT GGT GGT AAT TAT GAT AGA CTT GAA CAA CTT
Thr Phe Ala Phe Gly Gly Asn Tyr Asp Arg Leu Glu Gln Leu

1562                1577                1592
GCT GGT AAT CTG AGA GAA AAT ATC GAG TTG GGA AAT GGT CCA
Ala Gly Asn Leu Arg Glu Asn Ile Glu Leu Gly Asn Gly Pro

   1607                1622                1637
CTA GAG GAG GCT ATC TCA GCG CTT TAT TAT TAC AGT ACT GGT
Leu Glu Glu Ala Ile Ser Ala Leu Tyr Tyr Tyr Ser Thr Gly

        1652                1667                1682
GGC ACT CAG CTT CCA ACT CTG GCT CGT TCC TTT ATA ATT TGC
Gly Thr Gln Leu Pro Thr Leu Ala Arg Ser Phe Ile Ile Cys

            1697                1712                1727
ATC CAA ATG ATT TCA GAA GCA GCA AGA TTC CAA TAT ATT GAG
Ile Gln MET Ile Ser Glu Ala Ala Arg Phe Gln Tyr Ile Glu

                1742                1757
GGA GAA ATG CGC ACG AGA ATT AGG TAC AAC CGG AGA TCT GCA
Gly Glu MET Arg Thr Arg Ile Arg Tyr Asn Arg Arg Ser Ala
```

Figure 1 (Con't)

```
1772                1787                1802
 CCA GAT CCT AGC GTA ATT ACA CTT GAG AAT AGT TGG GGG AGA
 Pro Asp Pro Ser Val Ile Thr Leu Glu Asn Ser Trp Gly Arg

   1817                1832                1847
 CTT TCC ACT GCA ATT CAA GAG TCT AAC CAA GGA GCC TTT GCT
 Leu Ser Thr Ala Ile Gln Glu Ser Asn Gln Gly Ala Phe Ala

       1862                1877                1892
 AGT CCA ATT CAA CTG CAA AGA CGT AAT GGT TCC AAA TTC AGT
 Ser Pro Ile Gln Leu Gln Arg Arg Asn Gly Ser Lys Phe Ser

            1907                1922                1937
 GTG TAC GAT GTG AGT ATA TTA ATC CCT ATC ATA GCT CTC ATG
 Val Tyr Asp Val Ser Ile Leu Ile Pro Ile Ile Ala Leu MET

                1952                1967
 GTG TAT AGA TGC GCA CCT CCA CCA TCG TCA CAG TTT TCT TTG
 Val Tyr Arg Cys Ala Pro Pro Pro Ser Ser Gln Phe Ser Leu

1982                1997                2012
 CTT ATA AGG CCA GTG GTA CCA AAT TTT AAT GCT GAT GTT TGT
 Leu Ile Arg Pro Val Val Pro Asn Phe Asn Ala Asp Val Cys

   2027                2042                2057
 ATG GAT CCT GAG CCC ATA GTG CGT ATC GTA GGT CGA AAT GGT
 MET Asp Pro Glu Pro Ile Val Arg Ile Val Gly Arg Asn Gly

       2072                2087                2102
 CTA TGT GTT GAT GTT AGG GAT GGA AGA TTC CAC AAC GGA AAC
 Leu Cys Val Asp Val Arg Asp Gly Arg Phe His Asn Gly Asn

            2117                2132                2147
 GCA ATA CAG TTG TGG CCA TGC AAG TCT AAT ACA GAT GCA AAT
 Ala Ile Gln Leu Trp Pro Cys Lys Ser Asn Thr Asp Ala Asn

                2162                2177
 CAG CTC TGG ACT TTG AAA AGA GAC AAT ACT ATT CGA TCT AAT
 Gln Leu Trp Thr Leu Lys Arg Asp Asn Thr Ile Arg Ser Asn

2192                2207                2222
 GGA AAG TGT TTA ACT ACT TAC GGG TAC AGT CCG GGA GTC TAT
 Gly Lys Cys Leu Thr Thr Tyr Gly Tyr Ser Pro Gly Val Tyr

   2237                2252                2267
 GTG ATG ATC TAT GAT TGC AAT ACT GCT GCA ACT GAT GCC ACC
 Val MET Ile Tyr Asp Cys Asn Thr Ala Ala Thr Asp Ala Thr

       2282                2297                2312
 CGC TGG CAA ATA TGG GAT AAT GGA ACC ATC ATA AAT CCC AGA
 Arg Trp Gln Ile Trp Asp Asn Gly Thr Ile Ile Asn Pro Arg
```

Figure 1 (Con't)

```
            2327                2342                2357
TCT AGT CTA GTT TTA GCA GCG ACA TCA GGG AAT AGT GGT ACC
Ser Ser Leu Val Leu Ala Ala Thr Ser Gly Asn Ser Gly Thr

                2372                2387
ACA CTT ACA GTG CAA ACC AAC ATT TAT GCC GTT AGT CAA GGT
Thr Leu Thr Val Gln Thr Asn Ile Tyr Ala Val Ser Gln Gly

2402                2417                2432
TGG CTT CCT ACT AAT AAT ACA CAA CCT TTT GTG ACA ACC ATT
Trp Leu Pro Thr Asn Asn Thr Gln Pro Phe Val Thr Thr Ile

   2447                2462                2477
GTT GGG CTA TAT GGT CTG TGC TTG CAA GCA AAT AGT GGA CAA
Val Gly Leu Tyr Gly Leu Cys Leu Gln Ala Asn Ser Gly Gln

       2492                2507                2522
GTA TGG ATA GAG GAC TGT AGC AGT GAA AAG GCT GAA CAA CAG
Val Trp Ile Glu Asp Cys Ser Ser Glu Lys Ala Glu Gln Gln

            2537                2552                2567
TGG GCT CTT TAT GCA GAT GGT TCA ATA CGT CCT CAG CAA AAC
Trp Ala Leu Tyr Ala Asp Gly Ser Ile Arg Pro Gln Gln Asn

                2582                2597
CGA GAT AAT TGC CTT ACA AGT GAT TCT AAT ATA CGG GAA ACA
Arg Asp Asn Cys Leu Thr Ser Asp Ser Asn Ile Arg Glu Thr

2612                2627                2642
GTT GTC AAG ATC CTC TCT TGT GGC CCT GCA TCC TCT GGC CAA
Val Val Lys Ile Leu Ser Cys Gly Pro Ala Ser Ser Gly Gln

   2657                2672                2687
CGA TGG ATG TTC AAG AAT GAT GGA ACC ATT TTA AAT TTG TAT
Arg Trp MET Phe Lys Asn Asp Gly Thr Ile Leu Asn Leu Tyr

       2702                2717                2732
AGT GGG TTG GTG TTA GAT GTG AGG GCA TCG GAT CCG AGC CTT
Ser Gly Leu Val Leu Asp Val Arg Ala Ser Asp Pro Ser Leu

            2747                2762                2777
AAA CAA ATC ATT CTT TAC CCT CTC CAT GGT GAC CCA AAC CAA
Lys Gln Ile Ile Leu Tyr Pro Leu His Gly Asp Pro Asn Gln

                2792        2805        2815        2825
ATA TGG TTA CCA TTA TTT TGATAGACAG ATTACTCTCT TGCAGTGTGT
Ile Trp Leu Pro Leu Phe

      2835        2845        2855        2865        2875
ATGTCCTGCC ATGAAAATAG ATGGCTTAAA TAAAAAGGAC ATTGTAAATT
```

Figure 1 (Con't)

```
      2885       2895       2905       2915       2925
TTGTAACTGA AAGGACAGCA AGTTATTGCA GTCCAGTATC TAATAAGAGC

      2935       2945       2955       2965       2975
ACAACTATTG TCTTGTGCAT TCTAAATTTA TGGATGAATT GTATGAATTA

      2985       2995       3005       3015       3025
AGCTAATTAT TTTGGTCATC AGACTTGATA TCTTTTTGAA TAAAATAAAT

      3035       3045       3055       3065       3075
AATATGTTTT TTCAAACTTA TAAAACTATG AATGATATGA ATATAATGCG

      3085       3095       3105       3115
GAGACTAGTC AATCTTTTAT GTAATTCTAT GATGATAAAA GCTT
```

Figure 2

10 20
Ala Asp Val Cys Met Asp Pro Glu Pro Ile Val Arg Ile Val Gly Arg Asn Gly Leu Cys

30 40
Val Asp Val Arg Asp Gly Arg Phe His Asn Gly Asn Ala Ile Gln Leu Trp Pro Cys Lys

50 60
Ser Asn Thr Asp Ala Asn Gln Leu Trp Thr Leu Lys Arg Asp Asn Thr Ile Arg Ser Asn

70 80
Gly Lys Cys Leu Thr Thr Tyr Gly Tyr Ser Pro Gly Val Tyr Val Met Ile Tyr Asp Cys

90 100
Asn Thr Ala Ala Thr Asp Ala Thr Arg Trp Gln Ile Trp Asp Asn Gly Thr Ile Ile Asn

110 120
Pro Arg Ser Ser Leu Val Leu Ala Ala Thr Ser Gly Asn Ser Gly Thr Thr Leu Thr Val

130 140
Gln Thr Asn Ile Tyr Ala Val Ser Gln Gly Trp Leu Pro Thr Asn Asn Thr Gln Pro Phe

150 160
Val Thr Thr Ile Val Gly Leu Tyr Gly Leu Cys Leu Gln Ala Asn Ser Gly Gln Val Trp

170 180
Ile Glu Asp Cys Ser Ser Glu Lys Ala Glu Gln Gln Trp Ala Leu Tyr Ala Asp Gly Ser

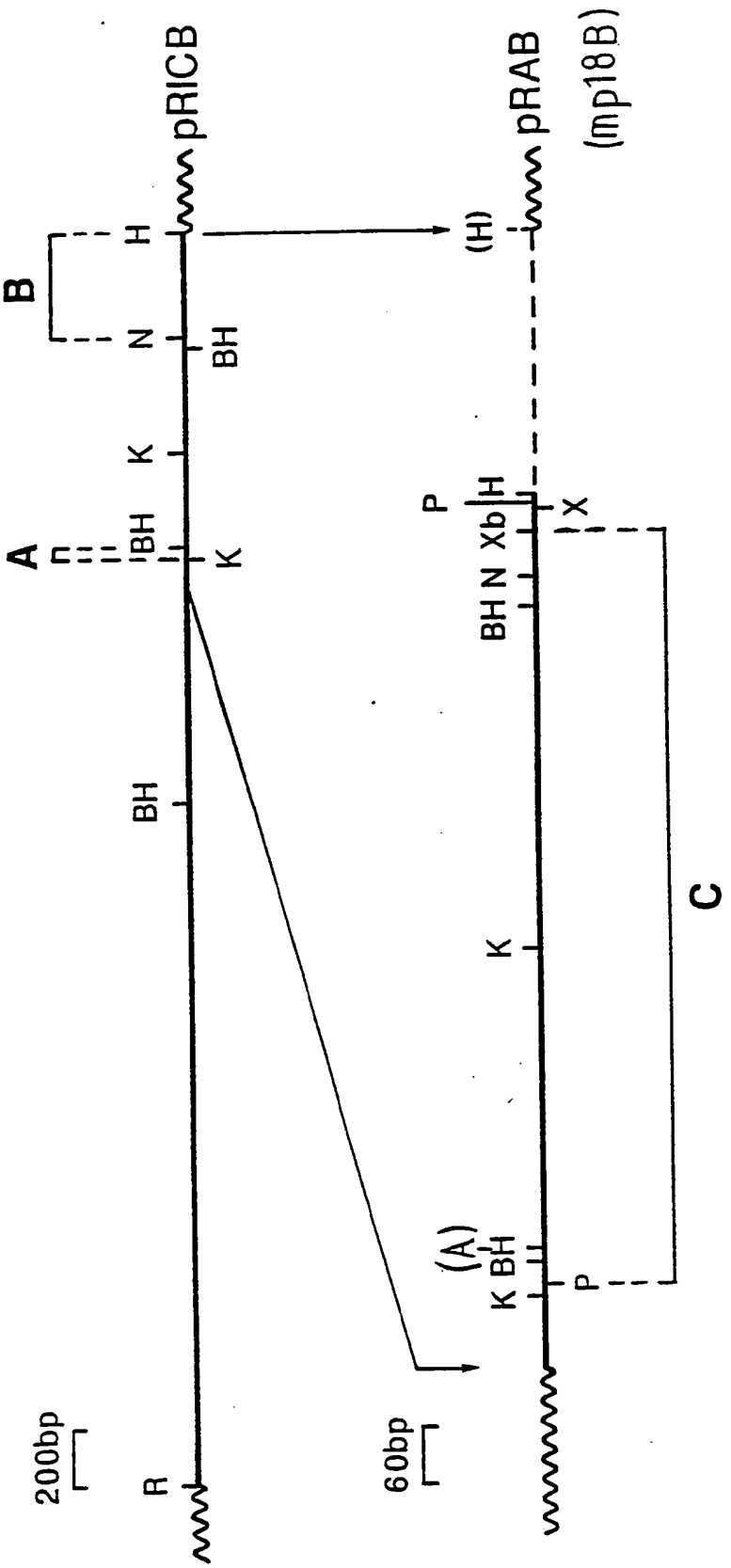
190 200
Ile Arg Pro Gln Gln Asn Arg Asp Asn Cys Leu Thr Ser Asp Ser Asn Ile Arg Glu Thr

210 220
Val Val Lys Ile Leu Ser Cys Gly Pro Ala Ser Ser Gly Gln Arg Trp Met Phe Lys Asn

230 240
Asp Gly Thr Ile Leu Asn Leu Tyr Ser Gly Leu Val Leu Asp Val Arg Ala Ser Asp Pro

250 260
Ser Leu Lys Gln Ile Ile Leu Tyr Pro Leu His Gly Asp Pro Asn Gln Ile Trp Leu Pro

262
Leu Phe




"Fig. 3"

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/04238

**I. CLASSIFICATION OF SUBJECT MATTER** (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C07K 13/00
U.S. Cl.: 530/377,

**II. FIELDS SEARCHED**

Minimum Documentation Searched [7]

| Classification System | Classification Symbols |
|---|---|
| U.S. | 530/377, 396; 435/70, 68, 172.2, 172.3 |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Chemical Abstracts database search; keywords = ricin D, Bchain, lectin, bind!, domain!, glycosyl!

**III. DOCUMENTS CONSIDERED TO BE RELEVANT** [9]

| Category * | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No. [13] |
|---|---|---|
| X<br>Y | European Journal of Biochemistry 148 pages 265-270 LAMB et al. Issued 15 April 1985 "Nucleotide sequence of cloned CDNA coding for preproricin" particularly figure 5 and page 268 last paragraph. | 1<br>11-28 |
| X<br>Y | Proceedings of the National Academy of Sciences, USA 84, page 5640-5644 CHANG et al. Issued August 1987 "Cloning and expression of recombinant, functional ricin B chain" particulary page 5644 last paragraph. | 1<br>11-28 |
| Y | The Journal of Biologicl Chemistry 262 (11) pages 5398-5403 MONTFORT et al. Issued 15 April 1987 "The three dimensional structure of ricin at 2.8 A" particularly figure 5 and pages 5400 5402 | 1,<br>11-28 |

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

**IV. CERTIFICATION**

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 24 January 1989 | 22 MAR 1989 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| ISA/US | Michelle Marks |

Form PCT/ISA/210 (second sheet) (Rev.11-87)

FURTHER INF RMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

I. Claims 1, 23 and 28 drawn to Ricin D, B chain polypeptides

II. Claims 2-6, 7 and 29 drawn to Domain 1 alpha muteins of Ricin D, B chain polypeptides -See Attachment

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. Telephone Practice-See Attachment

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No |
|---|---|---|
| Y | The EMBO Journal 4(3) pages 645-653 GOUGH et al Issued March 1985 "Structure and expression of the mRNA for murine granulocyte-macrophage coloning stimulating factor | 1, 11-28 |
| Y | DNA 3(6) pages 479-488 ZOLLER et al. Issued December 1984 "Oligonucleotide - Directed Mutagenesis: A simple method using Z oligonucleotide primers and a single - stranded DNA template | 1, 11-28 |
| Y | Nucleic Acids Research 10(20) pages 6487-6500 ZOLLER and SMITH Issued 25 October 1982 "Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any fragment of DNA" | 1, 11-28 |
| Y | Molecular and Cellular Biology 2(2) pages 161-170 OKAYAMA and BERG Issued February 1982 "High-Efficiency Cloning of Full-length cDNA" | 1, 11-28 |
| Y | Agricultural Biological Chemistry 49(4) pages 1175-1180 SHIMODA et al. Issued April 1985 "Effects of iodination on cytoagglutination by and toxicity of ricinus communis lectins" | 1, 11-28 |
| Y | The Journal of Immunology 138(9) pages 3339-3344 COLOMBATTI Issued 1 May 1987 "Identification and characterization of a monoclonal antibody recognizing a galactose-binding domain of the toxin ricin" | 1, 11-28 |
| Y | Agricultural and Biological Chemistry 41(10) pages 2041-2046 MISE et al. Issued October 1977 "Isolation and Characterization of Ricin E from Castor Beans" | 1, 11-28 |

III. DOCUMENTS C NSIDERED TO BE RELEVANT (C NTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No |
|---|---|---|
| T | Nature 326 pages 624-626 RUTENBER et al. Issued 9 April 1989 "Structure and evolution of rich B chain" | 1<br>11-28 |
| X<br>Y | Biochemica et Biophysica Acta 872(3) pages 277-285 ARAKI et al. Issued 15 August 1986 "The complete amino acid sequence of the B-chain of the Ricinus communis agglutinin isolated from large-grain castor bean seeds" | 1<br>11-28 |
| X | Journal of Biochemistry 100(3) pages 781-788 HATAKEYAMA et al. Issued September 1986 "Identification of the Tryptophan residue located at the low-affinity saccharide binding site of ricin D" | 14,22 |
| X<br>Y | Biochimica et Biophysica Acta 911(2) pages 191-200 ARAKI et al. Issued 30 January 1987 "The complete amino acid sequence of the B-chain of ricin E isolated from small-grain castor bean seeds. Ricin E is a gene recombination product of ricin D and Ricinus communis agglutinin". | 1,<br>11,23<br>1,<br>12-28 |
| Y | US, A, 4,689,401 Ferris Issued 25 August 1987 | 1,<br>11-28 |
| X<br>Y | EP O 237,676 PIATAK Issued 23 September 1987, Particularly Figures 14 and 15, pages 31-36 | 1<br>11-28 |
| Y,P | AGRICULTURAL BIOLOGICAL CHEMISTRY 52(7) pages 1771-1776 KIMURA et al. Issued July, 1988 "Isolation of Glycopeptides from Ricinus Communis Lectins" | 28 |
| Y | FEBS Letter 191(1) pages 121-124 ARAKI et al Issued October 1985 "Revised amino acid sequence of the B-chain of ricin D due to loss of tryptophan in the cyanogen bromide cleavage" | 19-22<br>25,26 |

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No |
|---|---|---|
| Y,P | Agricultural Biological Chemistry 52(4) pages 1021-1026 YAMASAKI et al Issued April, 1988 "Hydrophobicities of ricin D and its constituent polypeptide chains" | 1, 11-28 |
| Y | Agricultural Biological Chemistry 50(1) pages 151-155 MISE et al Issued January 1986 "Identification of tyrosyl residue present in the high affinity saccharide - binding site of ricin D" | 14, 25,26 |
| Y | Agricultural Biological Chemistry 51(4) pages 1225-1226 FUNATSU et al Issued April 1987 "Involvement of the B chain C-terminal region in the high-affinity saccharide binding site of ricin D" | 12,13 22,25 26,27 |
| X | US, A 1984260 (Evers et al) Issued 11 December 1934, See Entire Document. | 1-3 |
| X | Biological Abstracts, Volume 81, No. 2, Issued 15 January 1986 (Philadelphia, Pennsylvania, USA) Partridge et al, "Inhibitory effects of parathyroid hormone on growth of osteogenic sarcoma cells" Abstract No. 15489. | 1-3 |
| X | Endocrinology, Volume 118, No. 6 Issued June 1986 (Baltimore, Maryland, USA) MacDonald et al, "Parathyroid Hormone stimulates the proliferation of cells derived from human bone", pages 2445 2449, See entire document. | 4-6 |
| Y | Journal of Investigative Dermatology, Volume 86, No. 6, Issued 1 June 1986 (Baltimore Maryland, USA) Smith et al, "Effect of 1, 25-dihydroxyvitamin $D_3$ on the morphologic and biochemical differentiation of cultured human epidermal keratinocytes grown in serum-free conditions", pages 709-714, See entire document. | 1-3 |

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No |
|---|---|---|
| Y | Science, Volume 231, Issued 24 January 1986 (Washington, D.C., USA) Merendino et al, "A parathyroid hormone-like protein from cultured human keratinocytes" pages 388-396, See entire document. | 1-3 |
| Y | Biological Abstracts, Volume 82, No. 101. Issued 15 November 1986. (Philadelphia, Pennsylvania, USA) Bikle et al, "1, 25-dihydroxyvitamin $D_3$ production by human keratinocytes: Kinetics and regulation", Abstract No. 93161. | 1-3 |
| Y | Journal of Clinical Endocrinology and Metabolism, Volume 65, No. 1, Issued January 1987, (Baltimore, Maryland, USA) Wu et al, "Skin-derived fibroblasts respond to human parathyroid hormone like adenylate cyclase-stimulating proteins" page 105-109. See entire clamed. | 1-3 |
| Y | Vertebrate Endocrinology (Philadephia, Pennsylvania, USA) Issued 1985, Norris, "Calcuim and Phosphate Homeostasis", pages 315-335, especially page 322. | 1-3 |

Attachment to Form PCT/ISA/ 210, Part VI. 1. (continued)

The specification defines 8 separate domains of the Ricin D, B chain molecule (1 alpha, 1 gamma, 1 beta, 1 lambda, 2 alpha, 2 gamma, 2 beta, 2 lambda), yet fails to distinguish any structural or functional relatedness between these separate domains. A proposed change within or between each domain bears no relationship to a proposed change within another domain since structural and functional relationships between the domains are viewed as distinct. Therefore, Groups I, II, III and IV bear no relationship to each other. The invention defined by Group V, which is drawn to therapeutic compositions can be manufactured with other toxic molecules or by other methods of conjugation, and, therefore, is a separate invention from the unconjugated Ricin D molecules

Time Limit for Filing a Protest

Applicant is hereby given 15 days from the mailing date of this Search Report in which to file a protest of the holding of lack of unity of invention. In accordance with PCT Rule 40.2 applicant may protest the holding of lack or unity only with respect to the group(s) paid for.

Attachment to Form PCT/ISA/210, Part VI. 1.
Telephone approval:

$140 payment approved by Bruce Eisen on December 23, 1988 for Goup IV; charge to Deposit Account No. 07-1060. Counsel was advised that he has no right to protest for any group not paid for and that any protest must be filed no later than 15 days from the date of mai ling of the search report (Form 210).

Reasons for holding lack of unity of invention:

The invention as defined by Group I (claims 1, 23, and 28) is drawn to Ricin D, B chain molecules.

The invention as defined by Group II (claims 2-6, 7 and 29) is drawn to changes in Domain 1 alpha of the Ricin D, B chain molecule.

The invention as defined by Group III (Claims 8-10, and 30) is drawn to changes in Domain 2 gamma of the Ricin D, B chain molecule.

The invention as defined by Group IV (claims 11-22 and 24-27) is drawn to changes in both Domain 1 alpha and 2 gamma of the Ricin D, B chain molecule.

The invention as defined by Group V (claims 33-38) is drawn to therapeutic compositions of Ricin D conjugates and methods of conjugation.

Attachment to PCT/ISA/210 Part VI.
OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

III. Claims 8-10, and 30 drawn to Domain 2 gamma muteins of Ricin D, B chain polypeptides

IV. Claims 11-22 and 24-27 drawn to Domain 1 alpha and 2 gamma muteins of Ricin D, B chain polypeptides

V. Claims 33-38 drawn to therapeutic compositions of Ricin D conjugates.